PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
BO 42877		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 99/ 00621	06/10/1999	
Applicant		
CAMPINA MELKUNIE B.V. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   PROCESS FOR OBTAINING GROWTH FACTOR PREPARATIONS (TGF-BETA AND IGF-1) FROM MI LK PRODUCTS HAVING LOW MUTUAL CROSS-CONTAMINATION

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
BO 42877 Dek		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL99/00621	06/10/1999	~~06/10/1999~~ Δ IB
International Patent Classification (IPC) or national classification and IPC		
C07K14/495		
Applicant		
CAMPINA MELKUNIE B.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
04/05/2001	18.01.2002
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Heckl, K Telephone No. +49 89 2399 8430

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL99/00621

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-12	as originally filed				

Claims, No.:

1-15	as received on	24/12/2001	with letter of	24/12/2001	

Drawings, sheets:

1/1	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-7
	No:	Claims 8-15
Inventive step (IS)	Yes:	Claims 3-7
	No:	Claims 1,2
Industrial applicability (IA)	Yes:	Claims 3-15
	No:	Claims 1,2

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

D1: WO 92 00014 A (CLAR SARL) 9 January 1992 (1992-01-09) cited in the application

D2: EP-A-0 335 554 (UNILEVER PLC ;UNILEVER NV (NL)) 4 October 1989 (1989-10-04)

D3: BELFORD D.A. ET AL.: 'Platelet derived growth factor, insulin-like growth factors, fibroblast growth factors and transforming growth factor beta do not account the cell growth activity present in bovine milk' JOURNAL OF ENDORINOLOGY, vol. 154, no. 1, 1997, page 45-55 XP000913489

D4: EP-A-0 556 083 (SNOW BRAND MILK PROD CO LTD) 18 August 1993 (1993-08-18)

Novelty (Art.33(3) PCT):

D2 discloses a composition comprising TGF-beta and IGF-1 (see D2, page 4, line 39 to page 5, line 26; claims 1-12). The composition of D2 does not comprise 30 - 50% immunoglobulins on protein. Accordingly, the subject-matter of claims 8-13 and 15 is novel over D2.

However, the various fractions originating from bovine milk by applying cation-exchange chromatography and containing IGF-1 and/or TGF-beta as disclosed in D3 (see Results and Discussion, in particular Introduction, the last paragraph) anticipate the product according to present claims 8-15. D4, Results and Discussion, discloses various fractions containing lactoperoxidase and obtained by cation-exchange chromatography from milk and as such do not affect novelty of claims 8-15.

Taken together, the subject-matter of claims 8-15 is not novel.

Inventiveness (Art.33(3) PCT) and Industrial applicability (Art.33(4) PCT):

D3 is considered closest prior art. It discloses the concentration of cell growth activity present in whey including IGF-I and TGF-beta by a single step cation exchange

chromatography. The presence of lactoperoxidase and immunoglobulin in the major protein species isolated by this single step cation exchange procedure has also been discussed (see D3, Abstract, Introduction, Discussion).

Accordingly, it was the problem underlying claims 1 and 2 to adapt the general teaching of D3 in order to provide a method for extracting growth factors from a milk product comprising cation exchange chromatography wherein IGF-1, TGF-beta (claim 1) and lactoperoxidase (claim 2) are separated more or less from each other.

As the solution of the problem posed, claim 1 teaches the application of cation exchange chromatography and a hydroxyapatite column and elution with at least two eluents of increasing salt or pH, selected from phosphate buffer, sodium chloride and potassium chloride solutions in order to obtain two separate fractions (i) comprising IGF-1 at least 10 times the amount of TGF-beta and (ii) comprising TGF-beta at least 5 times the amount of IGF-I). Claim 2 teaches to further elute the hydroxyapatite column with an eluent as defined in claim 1 to obtain a fraction comprising lactoperoxidase.

D1 also discloses a process for extracting growth factors from colostrum comprising cation exchange chromatography and hydroxyapatite adsorption chromatography (see D1, page 4, line 17 to page 5, line 4). Hence, the method steps of claim 1 of the present application and of D1 appear identical.

In this context it is noted that D1 does not disclose that IGF-1 and TGF- beta and lactoperoxidase could be and are separated from each other to variable degrees (see page 5, lines 1-4). In contrast, the result of the procedure according to claim 1 (and claim 2) of the present application is claimed to lead to two (three) different fractions. Therefore, it must be concluded that claim 1 of the present application does not disclose the technical features which allow to obtain the desired result.

Accordingly, claim 1 does not meet the requirements of Art.33(4) and Art.6 PCT (lack of clarity). In addition, a claim which does not comprise the features of the invention (see below) cannot be considered to meet the requirements of Art.33(3) PCT, of course.

The same applies mutatis mutandis to the teaching of claim 2.

Claims 3 and 4 (as far as depend from claim 3) provide additional technical features which allow to obtain the result to be achieved according to claims 1 and 2. Therefore, these claims meet the requirements of Art.33(4) and 6 PCT. These claims are also considered inventive since none of the cited documents allows to conclude in an obvious way that the 2-step procedure of D1 can lead to the separation of IGF-I and TGF-beta as claimed in claim 1 of the present application by applying appropriate conditions, eventually followed by a further elution step to obtain lactoperoxidase.

The same applies to claims 5-7 as far as being depended on claims 3 and 4.

Re Item VII

Certain defects in the international application

Claims 1 and 2 lack clarity (Art.6 PCT), see above, Re Item V.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicants *(for all designated States except US)*: CAMPINA MELKUNIE B.V. [NL/NL]; P.O. Box 2100, NL-5300 CC Zaltbommel (NL). N.V. NUTRICIA [NL/NL]; P.O. Box 1, NL-2700 MA Zoetermeer (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: KIVITS, Marinus, Gerardus , Cornelis [NL/NL]; Eikenstraat 50, NL-5482 CC Schijndel (NL). HENDRICKS, Andor, Wilhelm, Joseph [NL/NL]; Toren 23, NL-6031 GG Nederweert (NL). MALLEE, Leonard, Franciscus [NL/NL]; Artillerieweg 11, NL-5403 PB Uden (NL).

(74) Agent: DE BRUIJN, Leendert C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/25276 A1

(54) Title: PROCESS FOR OBTAINING GROWTH FACTOR PREPARATIONS (TGF-BETA AND IGF-1) FROM MILK PRODUCTS HAVING LOW MUTUAL CROSS-CONTAMINATION

(57) Abstract: The present invention relates to a process for extracting transforming growth factor β (TGF-β) and insulin-like growth factor 1 (IGF-1) from a milk product, comprising the steps of: a) recovering a basic fraction from the milk product by means of cationic exchange chromatography; b) passing the fraction obtained in step a) over a hydroxyapatite column; c) eluting the hydroxyapatite column with appropriate eluents in such a way as to obtain two separate fractions, these fractions being: i) a fraction comprising IGF-1 in the substantial absence of TGF-β; ii) a fraction comprising TGF-β in the substantial absence of IGF-1. This process can comprise a further step d) in which a lactoperoxidase fraction can be obtained. The invention further relates to the products obtained with this process.

PROCESS FOR OBTAINING GROWTH FACTOR PREPARATIONS (TGF-BETA AND IGF-1) FROM MILK PRODUCTS HAVING LOW MUTUAL CROSS-CONTAMINATION

The present invention relates to a process for obtaining a fraction comprising transforming growth factor β (TGF-β) in substantial absence of insulin-like growth factor (IGF-1) and a fraction comprising IGF-1 in substantial absence of TGF-β from milk products (milk or whey).

It has been known for some time that milk products contain growth factors that can have a beneficial activity. These growth factors are present in very low concentrations in the milk product, which is why they are sometimes referred to as micronutrients. They can be characterised by their isoelectric point, which is relatively high compared to other milk proteins and their molecular weight. The present invention in particular concerns the growth factors TGF-β and IGF-1.

TGF-β is a multifunctional protein found in all mammalian tissues. Currently, five forms of TGF-β are known, β1 to β5. It has been implicated in the development, differentiation and growth of tissue and the control of immune system function and carcinogenesis. TGF-β can be isolated from natural sources (e.g. blood platelets), mammalian milk or colostrum or can be produced by recombinant cells.

IGF-1, an anabolic, i.e. growth promoting, growth factor, is a small protein (molecular weight about 7800) which plays an important role in bone metabolism. It has been shown to stimulate growth of cells in culture. Animal growth is also stimulated in pituitary deficient, normal and catabolic states. Kidney function is also improved. It can be produced using recombinant DNA technology, solid phase peptide synthesis, by isolating it from blood serum or from mammalian milk, e.g. bovine or human milk.

As described above, it is known that IGF-1 and TGF-β can be extracted from milk products such as milk or whey. However, with the methods that have been applied up to now, using an economically feasible process without many purification steps, it was only possible to obtain

a mixture of these growth factors. For some uses, more in particular certain therapeutical applications it has been found that it is preferred to use an IGF-1 rich fraction essentially free of TGF-β and a TGF-β fraction essentially free of IGF-1.

An example of such a therapeutical use is that described in a copending application in the name of the Applicants. This document describes the use of TGF-β for preparing a pharmaceutical composition for preventing damage of the intestinal mucosa as a result of chemotherapy or radiotherapy. In this case it has been found that IGF-1 interferes with the activity of TGF-β. According to this application it is therefore necessary to supply TGF-β in the substantial absence of IGF-1 to the patient. Up to now such relatively pure TGF-β was only available from recombinant DNA techniques or by an economically unfeasible process for the isolation from milk (multiple step isolation, US5221734). These products are rather expensive and would make the treatment mentioned above inaccessible for large groups of patients.

WO 9200994 and WO 9529933 describe processes for isolating a plurality of growth factors from milk or whey. As described above, it is not always desired to have a mixture of growth factors, because some growth factors can have a negative effect on the activity of other growth factors. WO 9529933 further has the disadvantage that an acidification is applied. This results in separation of the growth factors from the binding proteins and also inactivates lactoperoxidase. The binding factors help survive the growth factors during passage in the intestine, where digestive enzymes may degrade the growth factors resulting in (partial) loss of activity.

EP 489884 describes a process for obtaining a mixture of growth factors from colostrum by cationic exchange chromatography followed by adsorption chromatography on hydroxyapatite, recovering the fraction retained on the hydroxyapatite. It is described that by this method more than 50 % of all the growth factors is isolated. This document only refers to a mixture of growth factors and gives no clue to how the much higher level of immunoglobulins and the virtual absence of lactoperoxidase, as compared to milk and/or whey, influence the amount and the mutual contamination of IGF-1 and TGF-β in enriched growth factor preparations. Moreover, this document does not clarify whether the growth factors are still bound to binding factors.

US 5221734 describes a process to isolate a Milk Growth Factor (MGF) from milk or whey. This process requires many steps, including ionic exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and size exclusion chromatography, resulting in low yields of TGF-β. This makes this process economically unfeasible.

WO 9526984 relates to a process that includes a step wherein the milk product is heated to denature the lactoperoxidase. Thereafter the lactoperoxidase is separated from the composition, which increases the efficiency of the final purification of the growth factors. However, it is preferable to separate native lactoperoxidase, for commercial application as a natural preservative. Furthermore, it is desirable to increase the specific activity of the lactoperoxidase remaining after isolation of the growth factors.

It is an object of the present invention to provide a process for isolating TGF-β and IGF-1 from a milk product as relatively pure fractions (i.e. high proportion of one growth factor relative to the other growth factor) achieving a high yield of growth factors. It is a further object of the invention to provide these growth factors in a form which is suitable for oral administration. It is a further object of the invention to recover TGF-β and IGF-1 from milk products as relatively pure fractions and simultaneously recover native lactoperoxidase in a high yield.

According to the present invention, a process has been found to separate fractions rich in growth factors and containing binding factors, and at the same time produce a lactoperoxidase fraction with a high activity. The present invention relates to a process for extracting transforming growth factor β (TGF-β) and insulin-like growth factor 1 (IGF-1) from a milk product, comprising the steps of

a) recovering a basic fraction from the milk product by means of cationic exchange chromatography;

b) passing the fraction obtained in step a) over a hydroxyapatite column;

c) eluting the hydroxyapatite column with appropriate eluents in such a way as to obtain two separate fractions, these fractions being

   i) a fraction comprising IGF-1 in the substantial absence of TGF-β;

   ii) a fraction comprising TGF-β in the substantial absence of IGF-1.

These steps can be followed by a further elution step d) wherein the hydroxyapatite column is eluted with an appropriate eluent in such a way as to obtain

iii) a fraction comprising lactoperoxidase.

The milk product which is used as a starting material for the present invention can be any mammalian milk or a milk derivative that contains growth factors, such as cheese whey or casein whey. Preferably bovine milk or milk derivative is used. The milk can be subjected to a pretreatment such as mild pasteurization, and/or defattted using a centrifuge or a microfiltration step.

Preferably, the starting material is first subjected to a minimal heat treatment. This is advantageous because

1) in such a heat treatment a considerable proportion of the bacteria naturally occurring in milk are killed and
2) the denaturation of lactoperoxidase and other milk serum proteins is minimized.

A minimal heat treatment is understood to mean heating to 80 °C at the most, for not more than a few seconds.

Further, it is highly advantageous to strip the starting material of fat before subjecting it to the adsorption and elution steps. It has been found that after fat removal the column in which the cationic exchange resin is contained hardly becomes greased or clogged up during the step of adsorption to the cationic exchange resin. This prevents undue pressure build up in the column and unfavourable shortening of the adsorption cycles.

It is preferred to remove fat by microfiltration because this effects at the same time the reduction of the microbial contamination of the starting material. In this connection, microfiltration is understood to mean filtration with a filter having openings between 0.1 and 10 μm.

The cationic exchange resin used in step a) can be of any suitable type known in the field. It is preferred to use a cationic exchange resin of a mean particle size in excess of 100 μm and of a sufficient mechanical strength to resist high pressures. This has the advantage that the cationic

exchange resin is resistant to high liquid loads, while the binding capacity is maintained. This makes it possible to process large amounts of liquid in short time, which is required for an industrially applicable process. Examples of suitable cationic exchange resins are S-Ceramic Hyper D, SP-Toyopearl, SP-Sepharose FastFlow and SP-Sepharose Big Beads.

Preferably the cationic exchange resin is equilibrated by buffering with a phosphate buffer of a pH value of 5.5 to 7.5. Then the milk product is passed through a column with the cationic exchange resin, for instance by pumping, whereby microcomponents adsorb from the starting material onto the cationic exchange resin. To prevent microbial growth, these processes are normally carried out at a temperature of 4 to 7 °C. However, the viscosity at this temperature leads to an unacceptable pressure build-up. Therefore, the adsorption is preferably carried out at a temperature of 15 to 20 °C to lower the viscosity of the milk or milk derivative, whilst maintaining a relatively hygienic condition.

According to a preferred embodiment the starting material is pumped at a high surface velocity (more than 500 cm per hour) and at a high liquid load (100-600 bed volumes per hour) over a cationic exchange resin having a mean particle size of 100-300 μm, as described in US 5,596,082. According to this embodiment a process is realised which is highly favourable from an economic point of view, having outstanding industrial applicability.

After the adsorption step, it is preferred to rinse the cationic exchange resin column of any residual milk product (starting material) by washing with a salt (NaCl) solution buffered at a pH between 5.5 and 7.5 and having a salt concentration of 0.15 molar or less.

After adsorption of the desired components onto the ionic exchange resin, an elution step is carried out. Preferably the components are eluted with a salt solution buffered at a pH between 5.5 and 7.5, preferably at a pH of about 6.5. As the salt preferably sodium chloride or potassium chloride is used, but also other salts e.g. ammonium acetate can be used. This results in a fraction containing the desired TGF-β, IGF-1 and lactoperoxidase.

In step b) of the process the fraction obtained after ionic exchange chromatography is passed over a hydroxyapatite column. Hydroxyapatite is a crystallized tricalcium phosphate which is used as a substrate for the absorption of proteins. Industrially applicable hydroxyapatite resins

are Macroprep Ceramic Hydroxyapatite from Biorad and HA Ultrogel from Biosepra. Hydroxyapatite has unique separation characteristics due to both phosphate and calcium that can act as ligands. Only recently, hydroxyapatite material that can be applied on production scale became available. It is now used in several production scale protein recovery/purification processes.

According to this step of the present invention the milk fraction obtained in step a) is passed through the hydroxyapatite column, for instance by pumping, whereby microcomponents adsorb from the starting material onto the hydroxyapatite. The adsorption is preferably carried out at a pH greater than 5.5 and a phosphate concentration of 5 to 100 mmole/l.

After the absorption step the hydroxyapatite column is eluted sequentially with suitable eluting liquids. Possible eluents are phosphate buffers, sodium chloride and potassium chloride solutions. For the different fractions these eluents must have an increasing salt concentration. It is also possible to apply an increasing pH gradient. Other possible eluents are known to the person skilled in the art. It is preferred that the overall concentration range of the salt solutions used is between 0.01 to 1.0 M.

According to the invention, to obtain an IGF-1 enriched fraction the column is typically eluted with a phosphate buffer having a pH of 5.5 to 7 and a phosphate concentration of 0.05 to 0.2 M, preferably a pH of 6.0 and a phosphate concentration of 0.15 M. To obtain a TGF-β enriched fraction the column is subsequently eluted with a phosphate buffer having a pH of 5.5 to 7 and a concentration of 0.2 to 0.3 M, preferably a pH of 6.0 and a concentration of 0.25 M.

Overall, the present process results in a recovery of both IGF-1 and TGF-β of about 25 to 50 % compared to the amounts present in the starting material.

In a preferred embodiment of the invention a further elution step is carried out to recover a lactoperoxidase fraction. According to this embodiment the hydroxyapatite column is eluted with a phosphate buffer having a pH of 5.5 to 8 and a phosphate concentration of 0.3 to 0.5 M, preferably a pH of 7 and a phosphate concentration of 0.5 M. This results in a native

lactoperoxidase fraction with a high activity, which is an additional benefit of the present invention.

The fractions obtained according to the present invention can be separated further into their respective components by means of known methods. Examples of separation methods that can be used are ionic exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography.

The final products can be treated further by techniques known in the art, to remove salt therefrom and/or to concentrate them. For salt removal for instance ultrafiltration or gel filtration can be used. For concentrating the fractions can be lyophilised or spraydried.

The present invention also relates to the different fractions of growth factors obtained with the present process. The invention thus also comprises a product containing a TGF-β rich fraction essentially free of IGF-1, wherein the ratio TGF-β to IGF-1 is greater than 5, preferably greater than 50. This product in particular contains more than 200 μg TGF-β per gram protein and less than 40 μg IGF-I per gram protein, as determined by ELISA (Enzyme Linked Immuno Sorbent Assay). Generally, these fraction will contain 2000 μg TGF-β per gram protein at the most.

The invention further comprises a product containing an IGF-1 rich fraction essentially free of TGF-β, wherein the ratio IGF-1 to TGF-β is greater than 10, preferably greater than 100. This product in particular contains more than 50 μg IGF-1 per gram protein, and less than 10 μg TGF-β per gram protein. Typically, such a product contains 500 μg IGF-1 per gram protein at the most.

As described before, when applying a final extraction step a product can be obtained containing lactoperoxidase having at least 1200 Units per mg, as determined with the ABTS method, essentially according to Shindler et al. (1976), European Journal of Biochemistry 65, 325 - 331.

The IGF-and TGF-fractions further contain about 30 to 50 % immunoglobulins on protein. Their main function is to interact with harmful micro-organisms such as bacteria. This

prevents the micro-organism from entering the blood circulation system. This situation in particular occurs when the intestinal mucosa of the patient has been damaged as a result of treatment with chemotherapy.

The immunoglobulins can be isolated from milk of mammals which have been hyperimmunised against certain pathogens or they can be isolated from normal bovine milk or whey. With the present process, using normal cow's milk as a starting material, a preparation is obtained rich in immunoglobulins, comprising IgG and IgA. 30 to 50 % of the protein fraction consists of immunoglobulins of the type IgG and IgA.

The TGF-β and IGF-1 fractions obtained according to the invention contain binding factors which are released upon acidification. Thus the latent and active forms of both growth factors may be determined by e.g performing a growth factor specific ELISA in the presence or absence of an acid treatment of the sample, respectively. The binding factors fulfil a role in the modulation of the growth factor activity and may protect the growth factors during passage through the gastrointestinal tract

The IGF-and TGF-fractions obtained according to the invention can be used for several purposes, one of which is the use during chemotherapy and radiotherapy for treatment and/or prevention of damage to the intestinal mucosa.

The present invention is further illustrated by means of the following examples and Figure 1 which shows the identification of immunoglobulins in an IGF-1 rich fraction.
In the examples the following methods were used to analyse the products obtained.

Test kits for the determination of TGF-β and IGF-1 are commercially available. Test kit used: Quantikine® for determination of human TGF-β from R&D Systems.

TGF-ß is determined using a quantitative sandwich enzyme immunoassay technique (ELISA). A monoclonal antibody specific for human TGF-ß2 has been pre-coated onto a microplate. Human and bovine TGF-ß are identical so that the antibody will detect the bovine form. Standards and samples are pipetted into the wells and any TGF-ß present is bound by the immobilized antibody. Prior to this step, since the TGF-ß in milk is present in a latent form, it

is first activated by an acid treatment to determine the total TGF-ß concentration. This activation step is left out to determine the amount of active TGF-ß.

After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-ß2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of TGF-ß2 bound in the initial step. The colour development is stopped and the intensity of the colour measured.

TGF-ß in samples is expressed as µg/g protein.

IGF-1: test kit used: IGF-1 ELISA DSL-10-2800 from Diagnostic Systems Laboratories, Inc.

IGF-1 is also determined by an enzymatically amplified "two-step"sandwich-type immunoassay similar to TGF-ß. Samples, controls and prediluted unknowns are incubated in microtitration wells which have been coated with anti-IGF-1 antibody. IGF-1 in milk can be bound to binding proteins, and therefore, an activation step using acid similar to TGF-ß is used when determining total IGF-1 concentration. The amount of free IGF-1 is determined when the activation step is left out.

IGF-1 in samples is expressed as µg/g protein protein.

Protein

Protein in samples is determined with the Bradford method using Lactoferrin to make the standard curve.

Example 1: Isolation of IGF-1, TGF-β and lactoperoxidase from milk

An ion exchange chromatography (IEC) column having a diameter of 10cm was packed with 1L of a strong cation exchanger (SP Sepharose Big Beads, Pharmacia). The column was preconditioned using a phosphate buffer (pH 6.5 0.025 M phosphate). The fat fraction of the milk was removed by means of centrifugation and 360L of the resulting skim milk was passed over the column at room temperature at a flow rate of 100BVH (Bed Volumes per Hour). The column was washed with 5L of a 0.10M NaCl pH6.5 solution. The adsorbed proteins were then fractionated by subsequently eluting the column with:

a) 5L of a 0.24M NaCl solution, pH6.5

b) 5L of a 1.00M NaCl solution, pH6.5

Fraction a) contains predominantly Lactoperoxidase and is rich in IGF-1 and TGF-ß. Fraction b) is rich in angiogenin and lactoferrin. According to the results, fraction a) contains 9 g protein, including 7 g LP, 200µg IGF-1 and 1000µg TGF-ß. Then the eluted fraction a) is diluted 20 fold and loaded onto a column containing 0.5L Hydroxyapatite (Biorad ceramic HAP type I, 40µm). at 15BVH. The column is washed with a buffer containing 60mM phosphate pH 6.0. The adsorbed proteins were then fractionated by subsequently eluting the column with:

c) 0.15M phosphate pH 6.0

d) 0.25M phosphate pH 6.0

e) 0.50M phosphate pH 7.0

Fraction c) contains 100µg IGF-1 (150µg /g protein) and is low in TGF-ß (1µg TGF-ß/g protein). Fraction d) contains 660µg TGF-ß (1000µg/g protein) and is low in IGF-1 (5µg IGF-1/g protein). Fraction e) contains 7g LP (1200 Units/mg).

Example 2: Isolation of IGF-1, TGF-β and lactoperoxidase from cheese whey

800 L microfiltered cheese whey were loaded onto 1L of SP Sepharose Big Beads at 150BVH. After washing the column with 5L of a 0.10M NaCl pH6.5 solution. The adsorbed proteins were fractionated by subsequently eluting the column with:

f) 5L of a 0.24M NaCl solution, pH6.5

g) 5L of a 1.00M NaCl solution, pH6.5

Fraction f) contains predominantly Lactoperoxidase and is rich in IGF-1 and TGF-ß. Fraction g) is rich in angiogenin and lactoferrin. According to the results, fraction f) contains 8 g protein, including 6 g LP, 170µg IGF-1 and 150µg TGF-ß. Then the eluted fraction is diluted 20 fold and charged onto a column containing 0.5L Hydroxyapatite (Biorad ceramic HAP type I, 40µm). at 15BVH. The column is washed with a buffer containing 60mM phosphate pH 6.0. The adsorbed proteins were then fractionated by subsequently eluting the column with:

h) 0.15M phosphate pH 6.0

i) 0.25M phosphate pH 6.0

j) 0.50M phosphate pH 7.0

Fraction h) contains 80µg IGF-1 (120µg /g protein) and is low in TGF-ß (<1µg TGF-ß/g protein). Fraction i) contains 100µg TGF-ß (600µg/g protein) and is low in IGF-1 (8µg IGF-1/g protein). Fraction j) contains 6.5g LP (1200 Units/mg).

Example 3: Isolation of IGF-1, TGF-β and lactoperoxidase from milk using different IEC elution conditions

The purity of the IEC fractions can be further increased by eluting the column under more stringent conditions.

Under identical conditions to those described in example 1, an IEC column was loaded with skim milk. The column was washed with a 5L of a 0.15M NaCl/10mM ammoniumacetate pH 5.5 solution. The growth factor rich fraction was then eluted by passing 3.5L of a 0.28M NaCl/10mM ammoniumacetate pH 5.5 solution over the column.

Although the yield of growth factors and lactoperoxidase in this step is slightly lower, the specific activity of the growth factors present in this fraction is higher versus the fraction obtained with the conditions as described in example 1, i.e. 40µg IGF/g protein and 180µg TGF/g protein.

Example 4: Isolation of IGF-1, TGF-β and lactoperoxidase from milk using different hydroxyapatite elution conditions

The fractions bound on the hydroxyapatite column can also be separated using other elution conditions.

Under identical conditions to those described in example 1, the IEC eluate was loaded on the hydroxyapatite column and the hydroxyapatite column was washed with a buffer containing 0.12M NaCl/25mM phosphate pH7.0. The IGF-1 rich fraction was then eluted with a buffer containing 0.20M NaCl/25mM phosphate pH7.0 and thereafter the TGF-β rich fraction was obtained by eluting the column with a buffer containing 0.35M NaCl/25mM phosphate pH7.0. Then the lactoperoxidase was obtained by passing a solution containing 1M NaCl/25mM phosphate over the column.

The IGF-1 rich fraction contained 80µg IGF-1 (120µg/g protein) and is low in TGF-β (3µg TGF-ß/g protein). The TGF-ß rich fraction contained 500µg TGF-ß (1100µg/g protein) and is low in IGF-1 (1µg/g protein). According to this method 6.5g LP was obtained.

Example 5: Identification of Immunoglobulins in IGF-1 rich fraction

The product resulting from Example 1 was evaluated by SDS Page to identify and quantify immunoglobulins (see figure 1). A 15% polyacrylamide gel was run under reducing and denaturing conditions using Phastsystem equipment (Pharmacia).

Lane 1: IEC fraction.

Lane 2: bovine IgG.

Lane 3: IGF-1 rich fraction. LP: Lactoperoxydase; IgH: heavy chain of IgG; IgL: light chain of IgG.

The protein band denoted RNase was identified by N-terminal sequencing.

From the figure it can be seen that the IGF-1 rich fraction in lane 3 does not contain any LP. Based on the color intensities of the bands, the immunoglobulin concentration in this sample is between 30 and 50%. The other major protein component was identified as RNase.

Example 6 : Determination of latent and active forms of growth factors

Starting from milk , fractions were obtained after subsequent elutions over a cationic exchange and a hydroxyapatite column. These fractions were freezedried , solubilized in an appropriate buffer and then assayed with ELISA, essentially as described in the preceding text.

Part of the sample was used as is and part was acidified according to the testkit instructions. Protein in the samples was determined with the Bradford assay using lactoferrin as the calibration protein. The IGF-1 enriched fraction contained as is 75 microgram IGF-1/ g protein and after acidification 175 microgram IGF-1/ g protein. This means that 43% of the total IGF-1 activity is scored as free IGF-1 and 57% of the total IGF-1 activity is bound to binding proteins. By analogy, the TGF-β enriched fraction contained as is 7 microgram TGF-β/ g protein, whereas upon acidification 540 microgram TGF-β/ g protein was found. This demonstrates that almost 99% of TGF-β was present in the latent form.

Claims

1. Process for extracting transforming growth factor β (TGF-β) and insulin-like growth factor 1 (IGF-1) from a milk product, comprising the steps of

a) recovering a basic fraction from the milk product by means of cationic exchange chromatography;

b) passing the fraction obtained in step a) over a hydroxyapatite column;

c) eluting the hydroxyapatite column with appropriate eluents in such a way as to obtain two separate fractions, these fractions being

i) a fraction comprising IGF-1 in the substantial absence of TGF-β;

ii) a fraction comprising TGF-β in the substantial absence of IGF-1.

2. Process according to claim 1, further comprising step

d) eluting the hydroyapatite column with an appropriate eluent in such a way as to obtain

iii) a fraction comprising lactoperoxidase.

3. Process according to claim 1, wherein the eluent for obtaining fraction i) is a phosphate buffer having a pH of 5.5 to 7 and a phosphate concentration of 0.05 to 0.2 M and the eluent for obtaining fraction ii) is a phosphate buffer having a pH of 5.5 to 7 and a phosphate concentration of 0.2 to 0.3 M.

4. Process according to claim 2 or 3, wherein the eluent for obtaining fraction iii) is a phosphate buffer having a pH of 5.5 to 8 and a phosphate concentration of 0.3 to 0.5 M.

5. Process according to any of claims 1 to 4, wherein step a) is carried out by passing the milk product at a high surface velocity and a high liquid load through a column packed with the cationic exchange resin.

6. Process according to any of claims 1 to 5, wherein the milk product is any mammalian milk, preferably milk from which fat has been removed.

7. Process according to claim 6, wherein the milk product is cheese whey.

8. Product obtainable with the process according to any of claims 1 to 7, which contains TGF-β in the substantial absence of IGF-1.

9. Product according to claim 8, wherein the ratio TGF-β to IGF-1 is greater than 5, preferably greater than 50.

10. Product according to claim 9, which contains more than 200 μg TGF-β per gram protein and less than 40 μg IGF-1 per gram protein.

11. Product obtainable with the process according to any of claims 1 to 7 , which contains IGF-1 in the substantial absence of TGF-β.

12. Product according to claim 11, wherein the ratio IGF-1 to TGF-β is greater than 10, preferably greater than 100.

13. Product according to claim 12, which contains more than 50 μg IGF-1 per gram protein and less than 10 μg TGF-β per gram protein.

14. Product obtainable with the process according to claim 2 or 4, which contains lactoperoxidase with an activity of at least 1200 Units/mg.

15. Product according to any of claims 8 to 13, containing immunoglobulins.

16. Product according to claim 15, containing 30 to 50 % immunoglobulins on protein.

17. Product according to any of claims 8 to 13, containing binding factors for the growth factors, which can be released upon acidification.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 99/00621

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/495 C07K14/65 A23J1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A23J A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 869 134 A (CAMPINA MELKUNIE BV) 7 October 1998 (1998-10-07) column 1, line 3-5 column 2, line 15-32,57 -column 3, line 7 column 3, line 47 -column 4, line 1 column 4, line 54 -column 5, line 15,30-41 column 6, line 5-27; claims 1,9,10 ---	1-17
A	WO 92 00014 A (CLAR SARL) 9 January 1992 (1992-01-09) cited in the application page 1, line 5-12 page 3, line 5-10,17 -page 4, line 12 page 4, line 17 -page 5, line 4 page 5, line 26 -page 6, line 30 page 7, line 2-4,28 -page 8, line 10; claims 1-3,7 --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2000	19/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tallgren, A

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INTERNATIONAL SEARCH REPORT

Information on patent family members

:rnational Application No
PCT/NL 99/00621

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 23 July 2001 (23.07.01)	
International application No. PCT/NL99/00621	**Applicant's or agent's file reference** BO 42877
International filing date (day/month/year) 06 October 1999 (06.10.99)	**Priority date** (day/month/year)
Applicant KIVITS, Marinus, Gerardus , Cornelis et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

04 May 2001 (04.05.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Juan Cruz

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

10/089925

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To:

DE BRUIJN, Leendert C.
Nederlandsch Octrooibureau
Scheveningseweg 82
P.O. Box 29720
NL-2502 LS The Hague
PAYS-BAS

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

Date of mailing (day/month/year) 16 April 2002 (16.04.02)	
Applicant's or agent's file reference BO 42877	**IMPORTANT NOTIFICATION**
International application No. PCT/NL99/00621	**International filing date** (day/month/year) 06 October 1999 (06.10.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address

CAMPINA MELKUNIE B.V.
P.O. Box 2100
NL-5300 CC Zaltbommel
Netherlands

State of Nationality: NL
State of Residence: NL
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address

CAMPINA B.V.
P.O. Box 2100
NL-5300 CC Zaltbommel
Netherlands

State of Nationality: NL
State of Residence: NL
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

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[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Peter WIMMER Telephone No.: (41-22) 338.83.38

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Scheveningseweg 82
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NOTIFICATION OF THE RECORDING OF A CHANGE

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International application No. PCT/NL99/00621	**International filing date** (day/month/year) 06 October 1999 (06.10.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
MALLEE, Leonard, Franciscus Artillerieweg 11 NL-5403 PB Uden Netherlands	NL	NL
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
MALLEE, Leonard, Franciscus Bloemstraat 21 NL-3581 WC Utrecht Netherlands	NL	NL
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
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